

ORIGINAL ARTICLE

Iran J Allergy Asthma Immunol
September 2007; 6(3): 107-114

Interaction of CpG-Oligodeoxynucleotides with Toll Like Receptor 9 Induces Apoptosis and Modulates Metalloproteinase-2 Activity in Human Intestinal Epithelium

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Received: 28 December 2006; Received in revised form: 29 May 2007; Accepted: 3 July 2007

ABSTRACT

Recent reports have indicated different effects of immunostimulatory sequences containing CpG-Oligodeoxynucleotides (ODN) on various immune cells. However, the exact role of CpG-ODN in the human gut is unclear.

In the present study, we assessed potential effects of CpG-ODN on non lymphoid cell (intestinal epithelial cell line HT-29) on a dose-response and time-course basis. Intestinal epithelial cell line HT-29 was treated with CpG-ODN (CpG 2006) and lipopolysaccharide (LPS) at 5, 10, 25, 50 µg/ ml and 1, 5, 10 µg/ ml concentrations, respectively. Following treatments, dose- response and time-course cytotoxicity using a colorimetric method, Metalloproteinase-2 (MMP-2) activity (using gelatin zymography) and apoptosis (using annexin-v flowcytometry method) assays were performed. Chloroquine treatment was also used for its inhibitory effect on endosomal acidification process to verify specific CpG-ODN and Toll Like Receptor 9 (TLR9) interactions.

Cytotoxicity analysis of CpG-ODN showed that CpG-ODN increased significantly the proliferation of CpG-ODN treated cells, as compared to untreated cells, at concentrations of 10-25 µg/ml ($p < 0.05$). Overall MMP-2 activity analysis showed significant differences between treated and untreated cells. However, minimal changes were observed when MMP-2 activity was assessed per cell. Moreover, CpG-ODN treated cells demonstrated an increasing apoptosis rate of 0.8%, 6.46% and 14.21% at concentrations of 5, 10, 25 µg/ml, respectively.

Collectively, our data indicated that intestinal epithelial cell line HT-29 is highly responsive to CpG effect in vitro and exhibits modified activities. The direct CpG-ODN and TLR-9 interactions in HT-29 cells could provide new approaches in malignant tumor therapeutic strategies.

Key words: Apoptosis; CpG-ODN; Cytotoxicity; HT-29; LPS; MMP-2; TLR9

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INTRODUCTION

Recognition of cytidine phosphorothioate guanine oligonucleotides (CpG-ODN), which induce signaling

through Toll-like receptor 9 (TLR9) is an integral part of the innate immune system. CpG motifs which are common in bacteria, but not in mammalian DNA, are currently under investigation as adjuvants in therapy against infections and cancer.^{1,2}

CpG oligodeoxynucleotides (ODNs) are synthetic DNA sequences that mimic bacterial DNA and have potent immunostimulatory effects on dendritic cells, B cells and natural killer (NK) cells.³ Murine and human dendritic cells and B cells express high levels of TLR9 and respond to bacterial DNA on stimulation with synthetic CpG-ODNs *in vitro*.^{4,6}

Potential of CpG-ODNs as therapeutic agents and vaccine adjuvants has been demonstrated in animal models of infection disease, allergy and cancer and are currently undergoing clinical trials in humans.⁷

The vast majority of published studies on CpG-ODNs have explored its effect on various immune cell populations. Some studies have focused on effects of CpG-ODNs on expression of cytokines in different cells.⁸ It has recently been demonstrated that the administration of CpG-ODN through tape-stripped skin induced a Th1-type immune response and suggested the CpG-ODN for vaccinological applications.⁹

Induction of direct tumorotoxic responses by the cells of the innate immune system might help to induce antitumor reactions through different recognition and destructive pathways than those used by T cells. Macrophages, the effector cells of the innate immune system, can play an important role in antitumor immune reactions. While involved in tumor development and progression; macrophages can also be activated to destroy cancer cells via both cell contact-dependent and -independent mechanisms. In a recent study to determine the effect of CpG therapy on macrophage activation, Buhtoiarov et al. showed that cytotoxic macrophage, rather than T cells or NK cells, can also play a role in antitumor reactions against weakly immunogenic tumors during immunotherapy with CpG-ODN.¹⁰

However, a few investigators have reported direct effect of CpG-ODNs on non immune cells. Some studies revealed constitutive expression of mRNA for TLR9 in three human colonic epithelial cell lines, HT-29, Caco2 and T84.¹¹ Toll-like receptors (TLRs) are essential in the host defense against infections and so far 10 human TLRs (TLR1-TLR10) and one TLR-homolog (RP105) have been characterized.¹² Although studies in cell lines and murine colitis models strongly

suggest that TLRs including TLR9 play a role in recognition and response to bacterial products in the gut, the experimental results are conflicting.¹³⁻¹⁵ Anticancer agents ultimately kill cancer cells by inducing the apoptotic cell-suicide pathway. There is evidence that CpG-ODNs bear anti-proliferative properties. The effects appear to be mediated both by the down-regulation of IL-10 production and increased apoptosis.^{16,17} These findings provide a major part of the rationale to assess the proapoptotic and anti-proliferative potentials of CpG-ODNs.

Moreover, Matrix metalloproteinases (MMPs), a family of related proteinases, are responsible for degradation and remodeling of extracellular matrix. These enzymes are over expressed in many tumors and there is solid evidence that they play a key role in the malignant progression and invasiveness of many tumors.¹⁸ We assessed, therefore, the effects of CpG-ODNs on MMP-2 activity.

The aim of the present study was to investigate the direct effects of CpG 2006 on human colon carcinoma cell line HT-29 characteristic phenotypes as assessed by cytotoxicity, MMP-2 activity, and apoptosis.

MATERIALS AND METHODS

Cell Line

The intestinal epithelial cell line HT-29 was purchased from National Cell Bank of Pasteur Institute of Iran, Tehran, Iran.

CpG-ODN 2006 and 2006C

The CpG-ODNs sequences used in this study were selected according to published reports¹ and were commercially synthesized (AlphaDNA, Montreal, Canada). ODN 2006C was used as CpG control desoxyoligonucleotide.

LPS

Lipopolysaccharide was obtained from Sigma-Aldrich (Germany).

Cell Culture

The cell line HT-29 was seeded at an initial density of 10,000 cells / well in 96-well tissue culture plate. Cells were maintained in DMEM medium supplemented with 10% fetal calf serum, 100 u/ml penicillin, 100 µg/ml streptomycin, with 5% CO₂, at 37°C and saturated humidity.

Dose-response and Time-course Treatments

Triplicate, CpG-ODN 2006 preparations at concentrations of 5, 10, 25, 50 µg/ml were transferred to overnight cultured of HT-29 cells. Non-treated cells were used as controls. Cells were cultured for 24 hours, 48 hours and 72 hours and then subjected to colorimetric cytotoxicity assay. A sample of each of the medium was also used for gelatinase zymography.

We assessed effects of chloroquine (Daru pakhsh, Terhan, Iran) on CpG-ODN effects. Before treating the cells with CpG, they were treated to 10 µg /ml chloroquine for 30 minutes, then the cells were treated to various concentration of CpG-ODN (5,10,25,50 µg /ml) followed by incubation for 24 hours and then colorimetric cytotoxicity and gelatin zymography were performed. The effect of LPS on HT-29 cell line was also evaluated at concentrations of 1, 5, 10 µg/ml.

Colorimetric Cytotoxicity Assay

This method was carried out as described previously.¹⁹ After each experiment, cells were washed three times with ice-cold phosphate buffered saline (PBS), pH 7.2, followed by fixation in a 5 % formaldehyde solution. Fixed cells were washed three times and stained with 1 % crystal violet. The stained cells were washed, lysed and solubilised with 33.33 % acetic acid solution. The density of developed purple color was read at 580 nm using a conventional ELISA reader instrument.

Gelatinase Zymography

This technique is mainly used for the detection of gelatinases, MMP-2 and MMP-9. The zymography procedure was adopted according to our previous publication.²⁰ It is extremely sensitive because levels of 10 pg of MMP-2 can be detected. Briefly, protein-content adjusted (normalized) aliquots of conditioned media, in triplicate, were subjected to sodium dodecyl sulfate polyacrilamide gel electrophoresis (SDS-PAGE) containing 2 mg/ml gelatin. Electrophoresis was performed for 3 hours at a constant voltage of 80 volts. After electrophoresis, gels were gently washed three times with 2.5 % Triton X-100 solution to remove SDS. The gel slabs were then incubated at 37°C overnight in 0.1 M Tris HCL gelatinase activation buffer (pH 7.4) containing 10 mM CaCl₂. Staining was performed with 0.5 % coomassie brilliant blue followed by intensive destaining. MMP-2 proteolysis areas appeared as clear bands against a blue background in

the gels. Using a UVI pro gel documentation system (GDS-8000, Cambridge, UK), quantitative evaluation of both surface and intensity of lysing bands, on the basis of grey levels, were compared relative to non-treated control wells and expressed as Relative Expression of gelatinolytic activity. To determine the CpG mode of action, a set of treatments and analyses were performed on cell-free media.

Cell Apoptosis Assay

The cells were treated to various concentrations of CpG-ODN 2006 (5,10,25,50 µg/ml) and incubated for 24 hours and harvested cells were stained with FITC-cojugated annexin-v (IQ Products Germany) and propidium iodide. The cell death was assessed by flowcytometry. Apoptotic and total cells were counted by flowcytometry instrument (Partec PAS Germany). The results were expressed as percentage of apoptotic to necrotic cells.

Statistical Analysis

The differences in cell proliferation and gelatinase activity were compared using Kruskal-wallis Test. P values <0.05 were considered significant.

RESULTS

Cytotoxic Analysis of CpG-ODN and LPS

Figure 1 shows the proliferative response of epithelial cell line HT-29 to CpG-ODN at various concentrations (5, 10, 25, 50 µg/ml) and different times. The comparison of proliferative response between various concentrations showed a significant difference (p <0.05).



Figure 1. Panel A: HT-29 cell line as observed in tissue culture plate (X40).

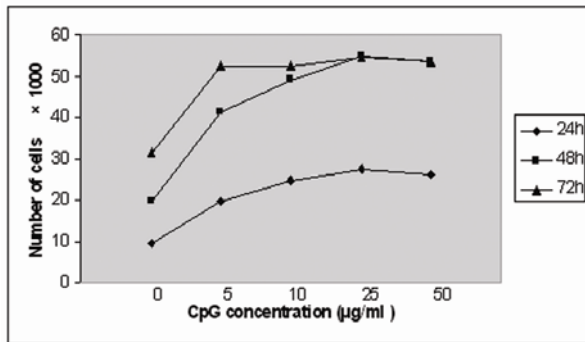


Figure 1. Panel B: Depiction of CpG cytotoxicity assay on HT-29 cell line.

HT-29 cell lines were cultured (Panel A) and subjected to CpG-ODN treatments (Panel B) at various concentrations (5-50 µg/ml). CpG-ODN cytotoxicity was analysed at 24, 48, and 72 hours, as indicated in Materials and Methods.

Figure 2 shows inhibitory effect of chloroquine on CpG-ODN. There was no significant difference between cytotoxic effect of CpG-ODN at given concentrations.

Figure 3 shows the proliferative response HT-29 cell line to LPS at concentrations of 1, 5, 10 µg/ml treatments for 24 h. The comparison of proliferative response between various concentrations showed a significant difference. Moreover, the comparison of proliferative response to LPS and CpG-ODN treated groups at equal concentrations showed significant difference at concentrations of 5 and 10 µg/ml ($p < 0.05$) (Figure 4).

In addition, there was a significant difference on proliferation effects between the ordinary CpG-ODN 2006 and CpG-ODN 2006C control (data not shown).

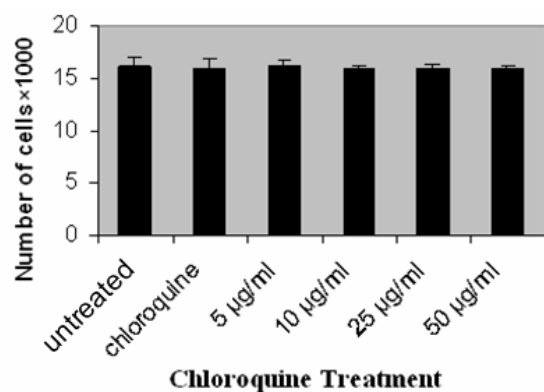


Figure 2. Inhibitory effect of chloroquine on CpG-ODN. HT-29 cell lines were treated with 10 µg/ml chloroquine (30 min); then were subjected to CpG-ODN at various concentration (5-50 µg/ml) and cytotoxicity was analysed at 24 hours.

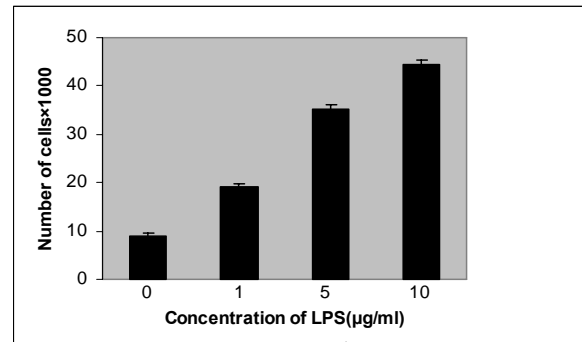


Figure 3. Cytotoxic effect of LPS on epithelial cell line. HT-29 cell lines were subjected to LPS at various concentrations (1-10 µg/ml) and cytotoxicity was analysed at 24 hours.

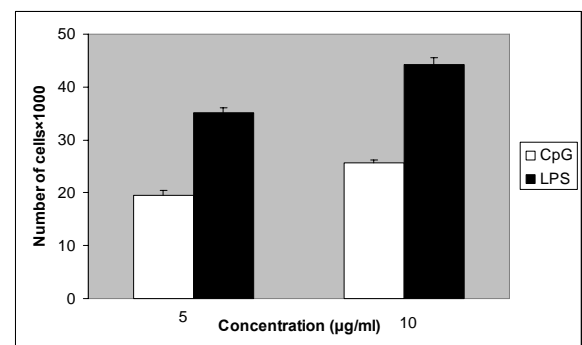


Figure 4. Comparison of cytotoxic effects of CpG-ODN and LPS. HT-29 cell lines were subjected to CpG-ODN and LPS at various concentrations (5-10 µg/ml) and cytotoxic effects of CpG-ODN and LPS were compared.

Effect of CpG-ODN and LPS on MMP-2 Activity

Dose response analysis of CpG-ODN on MMP-2 activity of epithelial cell line HT-29 is presented in figure 5. Overall MMP-2 activity analysis showed significant differences between different groups of treated and untreated cells ($p < 0.05$). However, minimal changes were observed when MMP-2 activity was assessed per cell. Assessments of cell-free media showed no difference in treatments versus untreated supernatant, indicating that CpG effects on cells are direct rather than on enzyme alone.

Effect of CpG-ODN on Apoptosis

As depicted in figure 6, the rates of apoptosis to necrosis for CpG-ODN at concentrations of 5, 10, 25 µg/ml were 0.8 %, 6.46%, 14.21%, respectively. These results show that apoptosis occurred significantly higher in CpG-ODN treated cells, as compared to untreated cells ($p < 0.05$).

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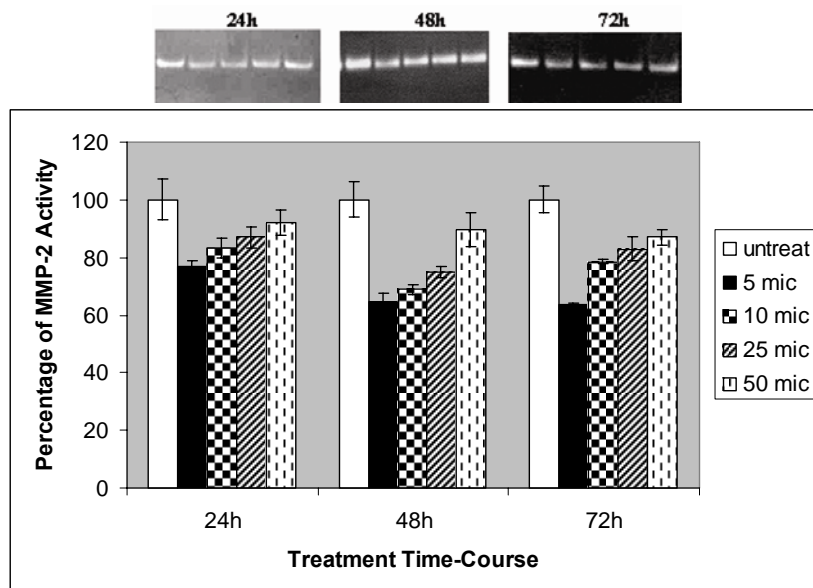


Figure 5. Dose response and time course analysis of CpG-ODN effect on MMP-2 activity. HT-29 cell lines were incubated with doses of CpG-ODN (0=untreated, 5, 10, 25, 50 $\mu\text{g/ml}$) for 24, 48, 72 hours, in triplicates, as described in "Materials and Methods". A representative zymogram gel for each treatment set was depicted on top of the histogram.

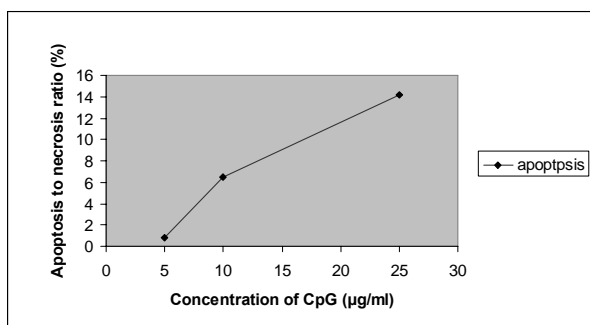


Figure 6. Assessment of apoptosis by flowcytometry. The apoptosis assay revealed that the rate of apoptosis as compared to necrosis was 0.8 %, 6.46 % and 14.21 % at concentrations of 5, 10 and 25 $\mu\text{g/ml}$, CpG-treatments, respectively. The number of apoptotic cells was demonstrated as percentage of apoptosis to necrosis in a given gated area.

DISCUSSION

Study of the antitumour properties of immunostimulatory CpG-ODN represent a rapidly expanding field of experimental and applied immunology.¹⁰

This study was aimed at determining the influence of CpG-ODN on epithelial cell line HT-29 that expresses TLR9 (specific receptor for CpG-ODN). The CpG-ODN 2006 was selected due to its strong immunostimulatory activity in human mononuclear

cells.²² Pedersen et al reported that CpG-ODN exposure induced no significant change in viability in colonic epithelial cells as judged by MTT metabolism.¹ In contrast, cytotoxicity analysis for CpG-ODN-treated cells in the present study revealed that CpG-ODN (CpG 2006) significantly increased proliferation of epithelial cell line HT-29 as compared to untreated cells. However, treatment of cells with CpG-ODN at concentration of 50 $\mu\text{g/ml}$ showed a lower proliferative response than 25 $\mu\text{g/ml}$ indicating that CpG-ODN might exhibit some cytotoxic potential at high dose administrations. A report on intestinal epithelial cells proliferation in proximal small intestinal biopsy specimens from children showed increased proliferation rates in a wide range of enteropathies for instance enteropathogenic E coli enteritis.²³

MMP-2 activity was also assessed in this study. One of the critical steps for tumor invasion and metastasis is the destruction of extracellular matrix, which is catalyzed mainly by the MMPs.²⁴ Thus; inhibition of MMPs could be beneficial in preventing tumor metastasis. Some studies found that MMP-2 was overexpressed in colorectal adenocarcinomas and weakly expressed in non cancer mucosa.²⁵ In another report Merrell et al. found that CpG oligonucleotide treatment decreased tissue inhibitor of metalloproteinase-3 expression and increased the levels of active MMP-13 in TLR9-expressing but not TLR9(-)

breast cancer cells without affecting MMP-8.²⁶ They also proposed a new molecular target for cancer therapy, since TLR9 has not previously been associated with cancer invasiveness. In a recent study regarding the TLR9 expression and function in human prostate cancer (CaP) cells, Ilvesaro et al. reported that treatment of TLR9(+), but not TLR9(-) CaP cells with CpG-ODNs increased their invasion, which was inhibited with chloroquine. CpG-ODN treatment also increased MMP-13 activity and neutralizing anti-MMP-13 antibody prevented CpG-ODN-induced invasion in TLR9(+) CaP cells.²⁷ The results of these reports might be in accordance with our presented data. As depicted in figure 5, overall MMP-2 activity analysis in our study showed significant differences between different groups of treated and untreated cells. However, minimal changes were observed when MMP-2 activity was assessed per cell, as elaborated in a dose response fashion. The CpG effect on MMP-2 activity indicates a very sensitive dose-dependent modulatory pattern, which calls for further investigations for verification of effective clinical applications. We performed paralleled paired zymography tests to determine whether CpG-ODN effects could also be detected in the absence of HT-29 cells. Interestingly, no direct chemical interaction was found between CpG and MMP-2 when HT-29 cells were depleted from the medium.

A number of recent reports indicate that TLR expression might have the potential to induce apoptosis upon ligand binding.^{28,29} It has also been demonstrated that CpG DNA inhibits spontaneous apoptosis of dendritic cells.³⁰ Some investigators showed that stimulation with CpG-ODN resulted in a strongly increased sensitivity of TLR9-expression in fibroblasts to apoptosis induced by staurosporine and UV-irradiation advocating that TLR9 signaling can sensitize fibroblasts for apoptosis.³¹ In contrast, rescue of both splenic B cells and WEHI-231 cells from apoptosis have been reported upon CpG treatments.³² Our data obtained from apoptosis assay showed induction of cell apoptosis by CpG-ODN. Further comparative analysis revealed that in the background of decreasing necrosis rate with higher CpG concentrations, the apoptosis to necrosis ratio is increased indicating that the relative number of apoptotic cells are increased at higher CpG concentrations (Figure 6).

In conclusion, intestinal epithelial cell line HT-29 is potentially highly responsive to CpG treatment in vitro and exhibits deviated cancer cell characteristic phenotypes.

Our data support the role of CpG in HT-29 cell function and provide a rationale for further clinical development of CpG therapy in patients with malignant intestinal tumors. The potential pro-apoptotic capacity of CpG therapy on colonic cancer HT-29 cell line is promising in further anti-cancer cell therapies. Moreover, studies on the mediators that have the potential to orchestrate onset of the mucosal inflammatory responses, T cell activation, and regulation of apoptosis signaling processes are also recommended.

ACKNOWLEDGEMENTS

This work has financially been supported by a grant from Medical Sciences/University of Tehran, 2006, Tehran, Iran.

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